The Plant Journal (2005) **44**, 840–853 doi: 10.1111/j.1365-313X.2005.02573.x

Transgenic proteoid roots of white lupin: a vehicle for characterizing and silencing root genes involved in adaptation to P stress

Claudia Uhde-Stone[1,2,4,*], Junqi Liu[1], Kelly E. Zinn[1,2], Deborah L. Allan[2] and Carroll P. Vance[1,3]
[1]*Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN 55108, USA,*
[2]*Department of Soil, Water, and Climate, University of Minnesota, 1991 Upper Buford Circle, St Paul, MN 55108, USA,*
[3]*USDA-ARS, Plant Science Research Unit, 1991 Upper Buford Circle, St Paul, MN 55108, USA, and*
[4]*Department of Biological Sciences, California State University East Bay, 25 800 Carlos Bee Blvd., Hayward, CA 94542, USA*

Received 22 May 2005; revised 20 August 2005; accepted 6 September 2005.
[*]For correspondence (fax 001 510 885 4747; e-mail claudia.stone@csueastbay.edu).

Summary

White lupin (*Lupinus albus* L.) has become an illuminating model for the study of plant adaptation to phosphorus (P) deficiency. It adapts to –P stress with a highly coordinated modification of root development and biochemistry resulting in short, densely clustered secondary roots called proteoid (or cluster) roots. In order to characterize genes involved in proteoid root formation and function in a homologous system, we have developed an *Agrobacterium rhizogenes*-based transformation system for white lupin roots that allows rapid analysis of reporter genes as well as RNA interference (RNA_i)-based gene silencing. We used this system to characterize a lupin multidrug and toxin efflux (*Lupinus albus MULTIDRUG AND TOXIN EFFLUX, LaMATE*) gene previously shown to have enhanced expression under –P stress. Here, we show that *LaMATE* had high expression in proteoid roots not only under –P, but also under –Fe, –N, –Mn and +Al stress. A portion containing the putative *LaMATE* promoter was fused to GUS and enhanced green fluorescence protein (EGFP) reporter genes, and a translational LaMATE::EGFP fusion was constructed under control of the *LaMATE* promoter. The *LaMATE* promoter directed P-dependent GUS and EGFP expression to proteoid roots. Confocal microscopy in white lupin and Arabidopsis point to the plasma membrane as the likely location of the LaMATE protein. LaMATE displayed homology to FRD3 in Arabidopsis, but did not complement an Arabidopsis ferric reductase defective 3 (FRD3) mutant. RNA_i-based gene silencing was shown to effectively reduce *LaMATE* expression in transformed white lupin roots. *LaMATE* RNAi-silenced plants displayed an about 20% reduction in dry weight.

Keywords: *Agrobacterium rhizogenes*, cluster roots, multidrug and toxin efflux (MATE), phosphorus deficiency, proteoid roots, white lupin.

Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development with P concentration ranging from 0.05 to 0.5% plant dry weight. Phosphorus plays a key role in many plant processes such as energy metabolism, photosynthesis, enzyme regulation, and the synthesis of nucleic acids and membranes (Raghothama, 1999). However, P is often unavailable for uptake by plants because it rapidly forms insoluble complexes with cations, particularly Al and Fe under acidic conditions. As a consequence, crop yield on 30 to 40% of the arable land of the world is limited by P availability (Runge-Metzger, 1995).

Plants have developed adaptive mechanisms that aid in the acquisition of P from soil. Compared with many plant species, white lupin (*Lupinus albus* L.) has been found to display extreme tolerance to P deficiency (Dinkelaker *et al.*, 1989; Gardner *et al.*, 1982). Its adaptation to low P is a highly coordinated modification of root development and biochemistry resulting in proteoid (or cluster) roots, which are densely clustered secondary roots of determinate growth (Johnson *et al.*, 1996b; Massonneau *et al.*, 2001; Neumann and Martinoia, 2002). Biochemical adaptations accompanying proteoid root formation include enhanced synthesis and exudation of acid phosphatase (Gilbert *et al.*, 1999; Neumann *et al.*, 2000) and the organic anions citrate and malate (Dinkelaker *et al.*, 1989; Neumann *et al.*, 1999). At the same time, the expression of a number of genes, including P

transporters and acid phosphatases, is strikingly enhanced in P-stressed proteoid roots (Liu *et al.*, 2001; Neumann *et al.*, 1999; Penaloza *et al.*, 2002; Uhde-Stone *et al.*, 2003). These adaptations greatly increase P uptake in proteoid root zones.

Because of its extreme tolerance for −P stress, white lupin has become an illuminating model for the study of the adaptation of plants to P deficiency. However, white lupin has been recalcitrant to transformation, though considerable effort has been devoted to the development of a transformation system for this model species. Here, we report the development of an *Agrobacterium rhizogenes*-based transformation system for white lupin. Transformation via *A. rhizogenes* led to the development of transgenic roots, including proteoid roots, on composite white lupin plants (plants with transgenic roots and non-transformed shoots) and thus has laid the foundation for the functional characterization of genes in transgenic white lupin roots.

A recent functional genomics approach identified 35 expressed sequence tags (ESTs) or EST contigs that showed enhanced expression of the corresponding genes in proteoid roots of P-deficient white lupin (Uhde-Stone *et al.*, 2003). Of special interest is a highly redundant EST that displayed strong induction in −P proteoid roots, compared with +P and −P normal roots. This contig showed homology to multidrug and toxin efflux (MATE) proteins. Multidrug and toxin efflux proteins are a large family of putative antiporters that are thought to be involved in the excretion of a variety of drugs and toxins (Brown *et al.*, 1999; Debeaujon *et al.*, 2001; Diener *et al.*, 2001; Hvorup *et al.*, 2003; Rogers and Guerinot, 2002,). So far only a few MATE genes have been cloned (Debeaujon *et al.*, 2001; Diener *et al.*, 2001; Rogers and Guerinot, 2002) and the function of most MATE proteins in plants remains elusive. In Arabidopsis, a MATE protein (FRD3) is hypothesized to be involved in shoot iron localization, possibly by transport of an iron chelator out of the xylem (Green and Rogers, 2004). We postulate that, in −P stressed proteoid roots of white lupin, *Lupinus albus* MULTIDRUG AND TOXIN EFFLUX (LaMATE) may be involved in the transport of small organic molecules as a response to nutrient stress.

The objectives of this research were: (i) to develop a system for the stable introduction of genes into white lupin; (ii) to isolate the *LaMATE* gene; (iii) to assess *LaMATE* promoter fidelity and cellular location of the encoded protein in a homologous system; and (iv) to evaluate RNA interference (RNA_i) of the MATE gene as a possible strategy for gene silencing in white lupin roots.

Results

Agrobacterium rhizogenes*-mediated transformation of white lupin roots*

The functional analysis of P-responsive genes in white lupin roots will be greatly facilitated by rapid introduction of chimeric gene constructs and analysis of roots from transformed plants. To that end, we have developed a protocol for *A. rhizogenes*-mediated transformation of white lupin. This procedure is based on a protocol developed by Boisson-Dernier *et al.* (2001) for transformation of *Medicago truncatula*. In general, transformation protocols that make use of *A. rhizogenes* involve the cotransfer of the root inducing (Ri) T-DNA and the T-DNA of a binary vector containing the transgene of interest. For the purpose of developing and optimizing the transformation methodology in white lupin, we used *A. rhizogenes* strain A4TC24, containing *LaMATE* promoter::GUS and enhanced green fluorescence protein (EGFP) fusions inserted between T-DNA borders of the binary plasmid pBI101.2 (BD Biosciences, Palo Alto, CA, USA). The transformation method involves the inoculation of sectioned seedling radicles (Figure 1a) with *A. rhizogenes* grown in the presence of 100 mM acetosyringone and 1% glucose to ensure optimal expression of the *A. rhizogenes* virulence genes (Cangelosi *et al.*, 1990). Inoculated seedlings were grown on slanted agar plates containing the appropriate nutrient solution (Figure 1b,c). Kanamycin (15 mg l^{-1}) was used to select for the cotransformation of transgenic roots (also referred to as 'hairy roots') directly with the gene constructs of interest (Figure 1d,e). Transformation efficiency and plant health were evaluated at different temperatures and light conditions. A growth temperature of 18–20°C at slightly dimmed light (200 photosynthetically active radiation, PAR) resulted in the most efficient transgenic root organogenesis and the subsequent development of white lupin 'composite plants' (Figure 1d). Mock inoculated control plants did not develop kanamycin-resistant roots at the wounded sections (Figure 1e). The transformation procedure resulted in approximately one out of two seedlings developing transgenic roots from the inoculated cut (Table 1). Transgenic root development started about 2 weeks after inoculation; developed proteoid roots were first found about 5 weeks after inoculation (Figure 1d). Transgenic roots had a similar morphology to normal roots (Figure 1f,g,i,j), except that transformed proteoid roots occasionally displayed secondary proteoid roots (Figure 1g,h, arrows), which were not observed on untransformed control plants.

All plants tested that were transformed with the GUS or EGFP reporter gene fusions and that had developed transgenic roots from inoculated sections displayed GUS or EGFP activity in their roots (Table 1). The generation of transformed roots in white lupin provides a homologous system to directly study gene expression and function in proteoid roots.

LaMATE *cDNA and homology to known multidrug and toxin efflux proteins*

We sequenced and analyzed the full-length complementary DNA (cDNA) clone corresponding to a previously described

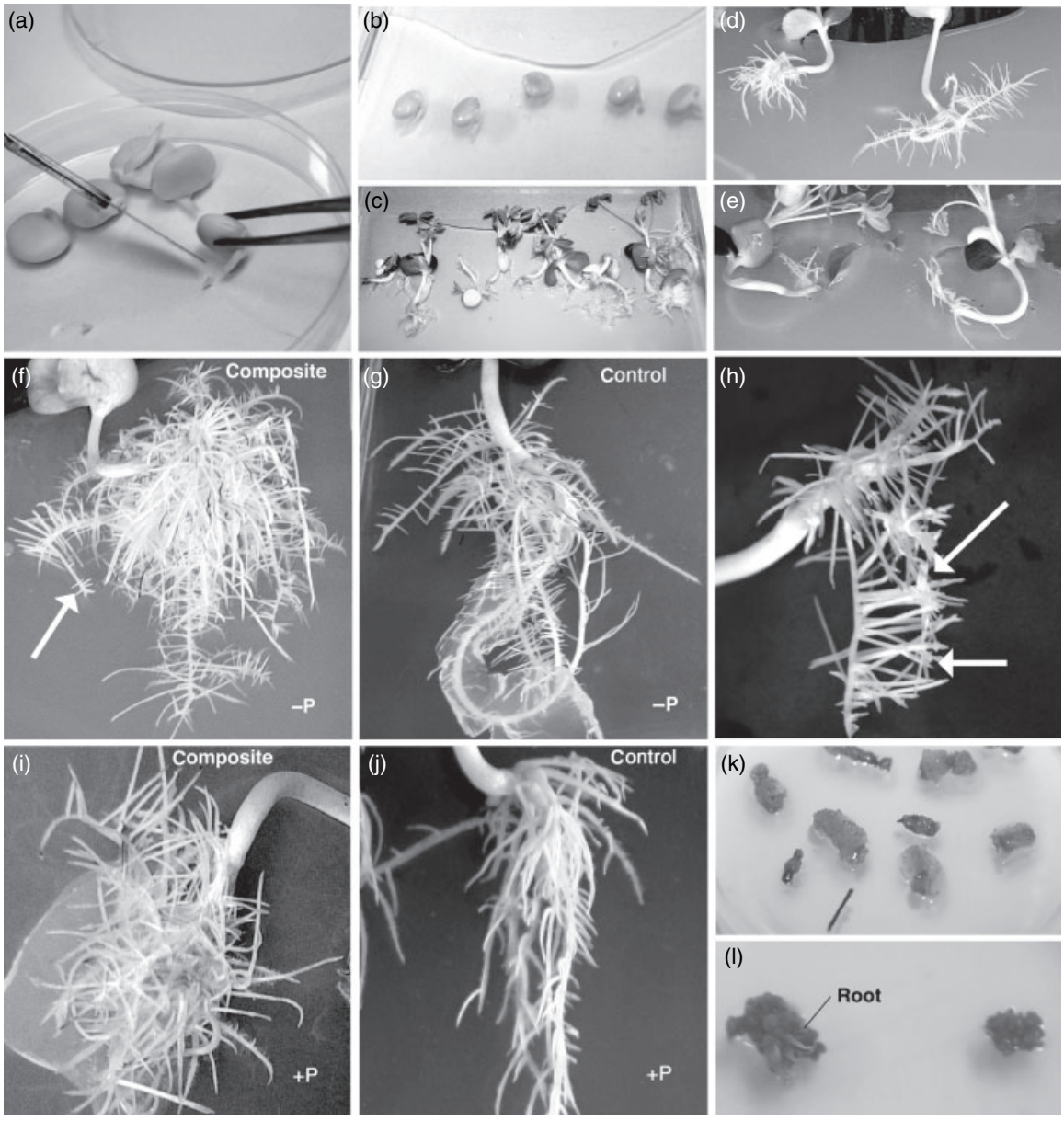


Figure 1. *Agrobacterium rhizogenes*-mediated transformation of white lupin roots.
(a) The tips of seedling radicles were removed, the wound inoculated with *A. rhizogenes*, and placed on slanted agarose plates containing Hoagland nutrient solution and kanamycin 15 (b).
(c) Transformed roots started to develop 3 weeks after inoculation.
(d) Fully developed proteoid roots were found about 5 weeks after inoculation and were clearly distinguishable from mock-inoculated control plants (e).
Transgenic roots (f and i) of white lupin composite plants had a morphology similar to non-transformed roots (g and j, grown without antibiotic) under –P (f and g) and +P (i and j) conditions, respectively; shown are roots of 7 week old lupin plants.
(f, h) Arrows: though proteoid roots were of similar morphology in transgenic and non-transformed control plants, transgenic lupin roots occasionally developed secondary proteoid roots, which were not observed in the non-transformed control plants.
(k) Sections of transformed proteoid roots develop into calli on nutrient plates containing sucrose and a 1:10 ratio of cytokin and auxin.
(l) Calli were transferred onto medium containing cytokinin: differentiation of a developing embryo and roots are visible.

EST (GenBank no. CA410182; Uhde-Stone *et al.*, 2003) with homology to MATE proteins. The 2.1-kb cDNA inserted in pBluescript contained a 1593 bp open reading frame (ORF) that encodes a deduced protein of 531 AA with an Mr of 56.9 kDa. The phylogram in Figure 2 displays the relationship of LaMATE and selected MATE proteins. The amino acid sequence displayed 55% identity and 75% similarity to the putative MATE protein FRD3 in Arabidopsis (At3g08040, GenBank accession no. NP_187461.1; Rogers and Guerinot, 2002), 51% identity with the FRD3-like protein FRDL

Table 1 Frequency of recovery of transgenic roots

Construct	Total plantlets inoculated	Number of plants with root growth from the inoculated cut (%)	Number of GUS/enhanced green fluorescence protein (EGFP) positive plants out of number tested (%)
Multidrug and toxin efflux (MATE)-Promoter::GUS	146	71 (49)	42 out of 42 (100)
MATE-Promoter::EGFP	60	34 (56)	20 out of 20 (100)
Control (mock-inoculated)	56	0 (0)	0 out of 6 (0)

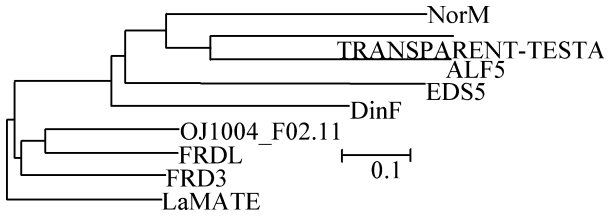


Figure 2. Phylogram of selected multidrug and toxin efflux (MATE) proteins. The phylogram was derived using the CLUSTALW program at the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/clustalw). Topological analysis of *Lupinus albus* MULTIDRUG AND TOXIN EFFLUX (LaMATE) indicates a membrane protein with 10 to 12 membrane spanning helices, depending on the program used for prediction (Mitaku and Hirokawa, 1999; Tusnady and Simon, 2001). LaMATE shares an enlarged cytoplasmic loop between transmembrane domains II and III with FRD3, FRD3-like (FRDL), OJ10004_F02.11 and EDS5. Sequence identity is particularly conserved at the putative transmembrane domains and at the C-terminus (when compared with FRD3 and FRDL), but is low over the whole length of the enlarged cytoplasmic loop.

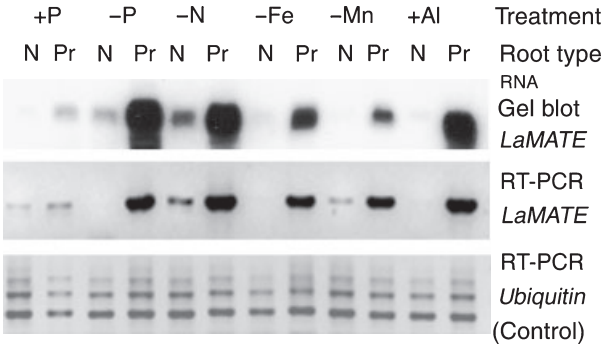


Figure 3. Gel blot analysis (upper panel) and RT-PCR (lower panels) of RNA isolated from normal (N) and proteoid (Pr) roots 14 days after emergence (DAE) of white lupin grown in the absence of P, N, Fe, Mn, or in the presence of Al.
Lupinus albus MULTIDRUG AND TOXIN EFFLUX (*LaMATE*) cDNA was used as a probe in the RNA gel blot analysis; each lane contains 15 μg of total RNA. RT-PCR was performed using primers to amplify *LaMATE* (middle panel) and *ubiquitin* (control, lower panel), respectively.

(At1g51340, GenBank accession no. NP_564588.2; Rogers and Guerinot, 2002) and 52% identity with OJ10004_F02.11 from *Oryza sativa* (GenBank accession no. NP_920452.1). In addition, a BLASTN search against ESTs in GenBank identified homologous ESTs in soybean (E-value 2E-75; GenBank accession no. BQ612651.1), *Medicago truncatula* (E-value 9E-47; GenBank accession no. BE997610.1) and *Lotus japonicus* (E-value 5E-36; GenBank accession no. CB829192.1). LaMATE displays higher similarity to the bacterial MATE protein damage-inducible protein F (DinF) (27%) than to enhanced disease susceptibility 5 (EDS5) (16%), TRANSPARENT TESTA 12 (15%) and aberrant lateral root formation 5 (ALF5) (14%) from Arabidopsis (Figure 2).

Effects of nutrient deficiencies and +Al stress on LaMATE *expression*

We have previously shown that *LaMATE* is highly expressed in −P stressed proteoid roots and to a lesser extend in −P normal roots; no expression was detected in leaves of −P or +P stressed lupin (Uhde-Stone *et al.*, 2003). To investigate whether expression of *LaMATE* in lupin is influenced by other nutritional stresses besides −P, RNA gel blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) were performed using RNA samples of normal and proteoid roots from white lupin grown in the absence of P (−P), iron (−Fe), nitrogen (−N), manganese (−Mn) and in the presence of aluminum (+Al), respectively. Figure 3 shows increased *LaMATE* mRNA in proteoid roots of plants grown under all nutrient stresses tested, as compared with normal roots and nutrient sufficient controls.

LaMATE *gene*

To better understand the genetic regulation of *LaMATE*, we isolated the encoding gene including the 5′ upstream putative promoter region. DNA gel blot analyses were performed to determine gene copy numbers and genomic organization. Under high stringency conditions, two hybridizing bands were obtained when white lupin genomic DNA was digested with the restriction enzyme *EcoR*I and hybridized against *LaMATE* cDNA (data not shown). The two fragments are due to an internal *EcoR*I site. This finding indicates that *LaMATE* is present as a single gene in the white lupin genome. In order to isolate the gene, the full-length *LaMATE* cDNA was used as a probe to screen a lupin genomic library. We identified two overlapping clones, which together contained 17 kb, including the entire coding region as well as approximately 5 kb upstream of the translation start codon.

About 9.3 kb of the 17-kb region was sequenced. A comparison of the nucleotide sequence of cDNA and genomic clones revealed identical coding regions. Eleven introns comprising 4187 bp were located between 12 exons comprising 2054 bp (Figure S1a). In comparison, FRD3 in Arabidopsis displays 13 exons and 12 introns, with an unusually large intron of approx. 2.6 kb in the 5′ untranslated region. As shown in Table 2, the *LaMATE* promoter region contains a number of putative *cis*-acting elements that have been implicated in nutrient deficiency and general stress response, including a phosphate starvation response 1 (PHR1) element (Rubio *et al.*, 2001), nitrogen regulatory gene 2 (Nit 2) elements (Fu and Marzluf, 1990), a motif similar to iron deficiency responsive cis-acting elements (IDE; Kobayashi *et al.*, 2003) and helix loop-helix elements (Blackwell and Weintraub, 1990). Figure S1(b) shows the distribution of putative *cis*-acting elements in the *LaMATE* promoter region.

Reporter gene fusions

To further investigate the spatial and temporal expression pattern of *LaMATE*, a 5-kb portion upstream of the *LaMATE* gene containing the putative promoter was ligated to GUS and EGFP reporter genes, respectively. In addition, a translational MATE–EGFP fusion under the control of the *LaMATE* promoter region was constructed to analyze protein localization. These constructs were used to analyze expression and protein localization in transgenic lupin and Arabidopsis roots.

The GUS expression pattern driven by the *LaMATE* promoter was monitored by histochemical GUS analysis. As demonstrated in Figure 4, GUS staining was found in nutrient stressed proteoid roots and location of GUS activity varied depending on the stage of proteoid root development. The *LaMATE* promoter drove expression of the GUS reporter gene at sites of newly forming proteoid root meristems (Figure 4a–c). As the proteoid roots elongate, GUS activity disappeared from the base and appeared in the stele of the proteoid rootlets (Figure 4e,f). At this developmental stage, GUS staining was most pronounced in the endodermis and/or pericycle, but not in the vascular system. With further development of the proteoid root, GUS activity continued to increase and eventually stained all tissues of the proteoid rootlets including root hairs (Figure 4g), but remained most pronounced in the stele (Figure 4g insert). A similar pattern of GUS activity was observed under Fe-deficiency (Figure 4h,i) and N-deficiency (Figure 4j). The staining pattern of root tips and adjacent zones varied in white lupin, but often displayed an unstained area at the tip (Figure 4h–j). A comparison of GUS staining intensity under −P, −Fe and −N-stress and +P control showed intense staining in fully developed proteoid roots of nutrient deficient plants, while proteoid roots of plants grown with sufficient nutrients displayed no or only very slight GUS activity (Figure 4k). These findings were confirmed in quantitative 4-methylumbelliferryl-b-D-glucuronide (MUG) assays, which revealed strongest MUG activity under Fe deficient conditions (Figure 5). Transgenic Arabidopsis harboring *LaMATE*-GUS directed expression in roots; staining was most pronounced at the transition between the differentiation and elongation zone behind the root tip (Figure 4l). In contrast to white lupin, expression of the *LaMATE*::GUS reporter gene fusion did not respond to nutrient-deficiency in Arabidopsis (data not shown), indicating that reporter gene studies in heterologous systems may lead to spurious conclusions.

Enhanced green fluorescence protein expression driven by the *LaMATE* promoter was analyzed in white lupin and Arabidopsis, using confocal laser scanning microscopy. Enhanced green fluorescence protein activity patterns corresponded to those observed with histochemical GUS analysis in both species (Figure 6a–d,h,i). A translational LaMATE::EGFP fusion displayed tissue-specific localization of the LaMATE protein (Figure 6c,d,h,i) similar to the expression pattern observed with GUS (Figure 4) and free EGFP (Figure 6a,b,g). Accordingly, translational LaMATE-EGFP directed fluorescence in white lupin was found mainly in proteoid root meristems and in the stele of proteoid rootlets (Figure 6c,d).

Table 2 Putative *cis*-acting elements in the promoter region of *Lupinus albus* MULTIDRUG AND TOXIN EFFLUX (LaMATE)

Name of element	Sequence	Position	Reference
Phosphate starvation response 1 (PHR 1) element	GNATATNC	−1833	Rubio *et al.*, 2001
Helix-loop-helix element	CAT(/G)A(/C)TG	−1930, −1424, −488	Blackwell and Weintraub, 1990
Vegetative storage protein B (VspB) box II (P_i response)	ATTAATT	−1878, −1559, −1146, −1027, −829, −809, −305	Tang *et al.*, 2001
DNA binding with one finger (Dof) elements	ACTTTA	−1136	Yanagisawa, 2004
Nitrogen regulatory protein 2 (Nit2) elements	TATCA(/T)A(/T)	−748, −41	Fu and Marzluf, 1990
Similar to iron-deficiency-responsive cis-acting elements (IDE)	CGACATGACTCTTAT *LaMATE* promoter CAAGCATGCTTCTTGC IDE consensus	−245	Kobayashi *et al.*, 2003

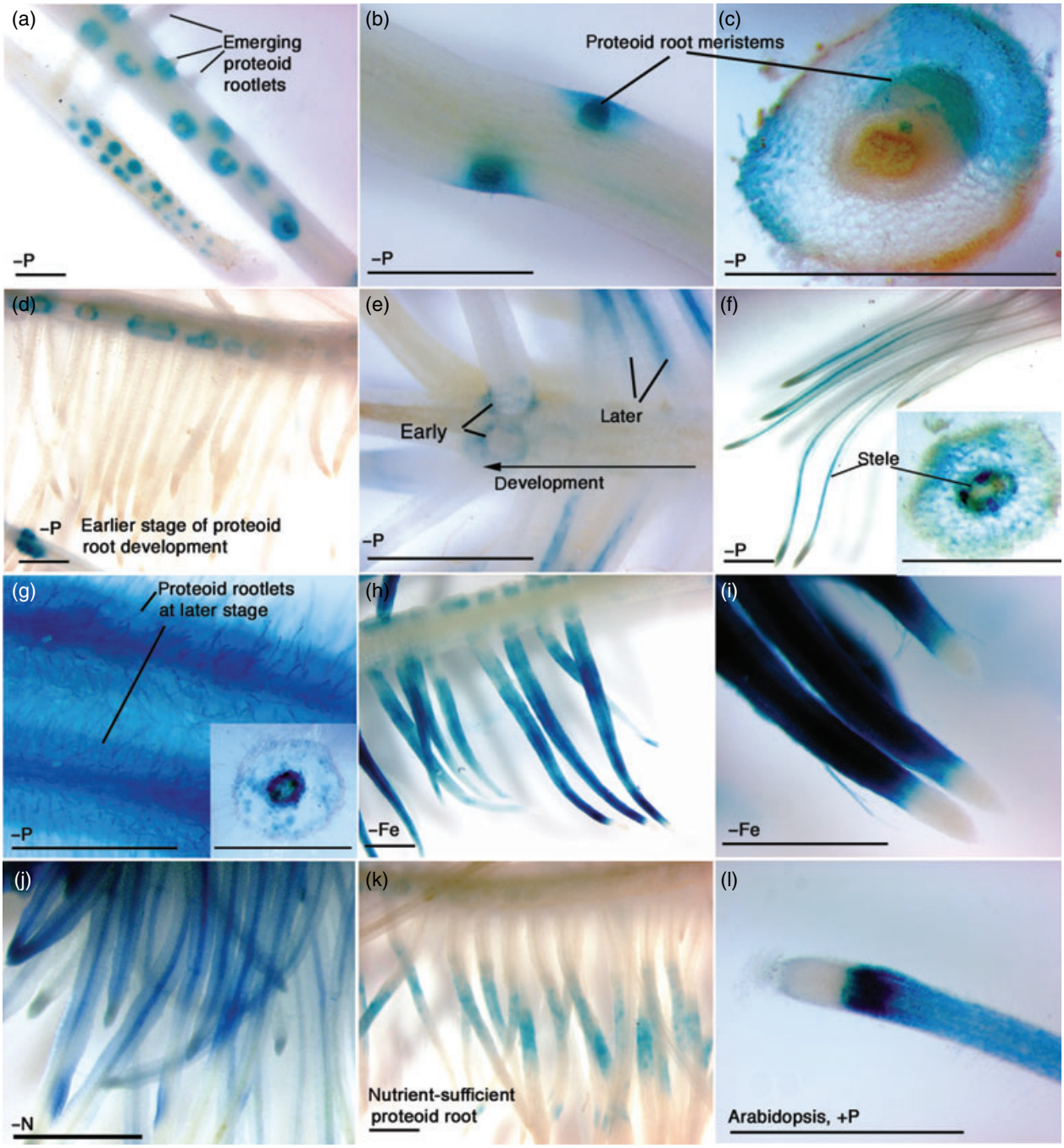


Figure 4. Histochemical localization of GUS activity directed by a *Lupinus albus MULTIDRUG AND TOXIN EFFLUX* (*LaMATE*) promoter::GUS fusion in transgenic roots of lupin (a–k) and Arabidopsis (l).
(a–g) Roots of P-deficient white lupin at different stages of development.
(a–c) The *LaMATE* promoter drives expression of the GUS reporter gene at newly forming meristems (a, b). Transsections of meristematic tissue displayed GUS activity in the epidermis and cortex surrounding the forming meristem (c). Rootlets just emerging from these meristems do not display GUS activity (a).
(d–g) Developing proteoid roots: GUS activity is first not apparent in the emerging lateral rootlets except at the base (d). During further proteoid root development, blue bases disappear and GUS activity appears in the stele (e, f). Transsection of rootlets that displayed GUS activity in the stele showed GUS staining mainly in endodermis and/or pericycle, but not in the vascular systems (f insert). After further maturation, GUS activity was found in all tissues, including root hairs (g), but remained most pronounced in the stele (g insert).
Similar patterns of GUS activity were observed under Fe-deficiency (h and i) and N-deficiency (j), accumulation in the stele was also found under these deficiencies (data not shown).
(k) Proteoid rootlets of plants grown under sufficient nutrients displayed no or only slight GUS activity.
(l) Transgenic Arabidopsis harboring *LaMATE*-GUS directed expression in roots, most pronounced at the elongation zone behind the root tip. Expression of the *LaMATE*::GUS reporter gene fusion did not respond to nutrient-deficiency in Arabidopsis. Bar size = 1 mm, in insert f and g = 0.5 mm.

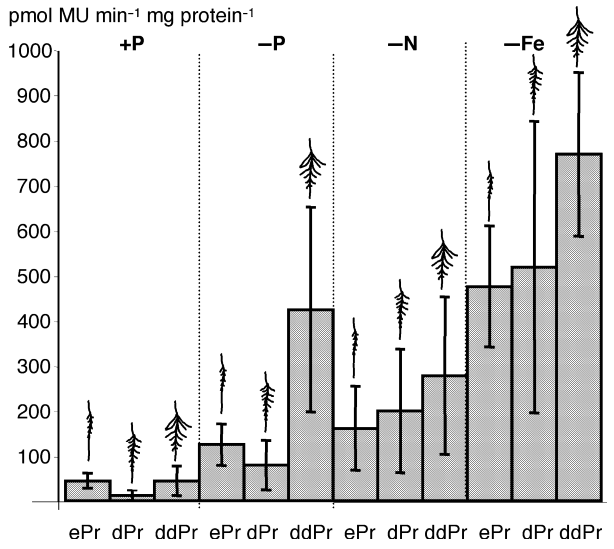


Figure 5. MUG assay of proteoid roots at different stages of development under sufficient (+P) and deficient (–P), (–N), (–Fe) nutrients, respectively. The MUG assays confirm the highest expression of *Lupinus albus MULTIDRUG AND TOXIN EFFLUX* (*LaMATE*) in fully developed proteoid roots under nutrient deficiency. Pr, proteoid roots, e, emerging, d, developing, dd, developed.

Confocal oil immersion microscopy was performed to assess subcellular localization of EGFP activity. Epidermal cells showed free EGFP, driven by the *LaMATE* promoter, in the nucleus and cytoplasm of transgenic white lupin (Figure 6e) and Arabidopsis (Figure 6g), respectively. Propidium iodide staining (red) of cell walls and nucleotide, coupled with plasmolysis experiments of lupin root cells, showed that translationally fused EGFP fluorescence associated with the membrane of plasmolysed cells (green), rather than with the cell wall (yellow–red; Figure 6f). Propidium iodide stained Arabidopsis roots expressing the LaMATE EGFP fusion (Figure 6j), compared with a known plasmamembrane-localized EGFP fusion [Figure 6k; Cutler *et al.*, 2000; available as no. CS84758 at the Arabidopsis Biological Resource Center (ABRC)] and a known tonoplast-localized EGFP fusion (Figure 6l; Cutler *et al.*, 2000; ABRC no. CS84727), respectively, reveals similarity of the GFP pattern between the LaMATE::EGFP fusion and the plasmamembrane-located EGFP fusion. This similarity indicates a plasmamembrane location of LaMATE; circular vacuolar membranes that were frequently observed in the tonoplast-located control (Figure 6l) were not observed with the LaMATE::EGFP fusion. In addition, no inclusions of the fluorescent outline at the site of the nucleus (stained red), typical for a tonoplast location of the EGFP fusion, were observed in lupin or Arabidopsis. Taken together, these observations in lupin and Arabidopsis indicate a likely subcellular location of the LaMATE protein in the plasmamembrane.

Analysis of LaMATE *in an Arabidopsis T-DNA insertion line of At3g08040*

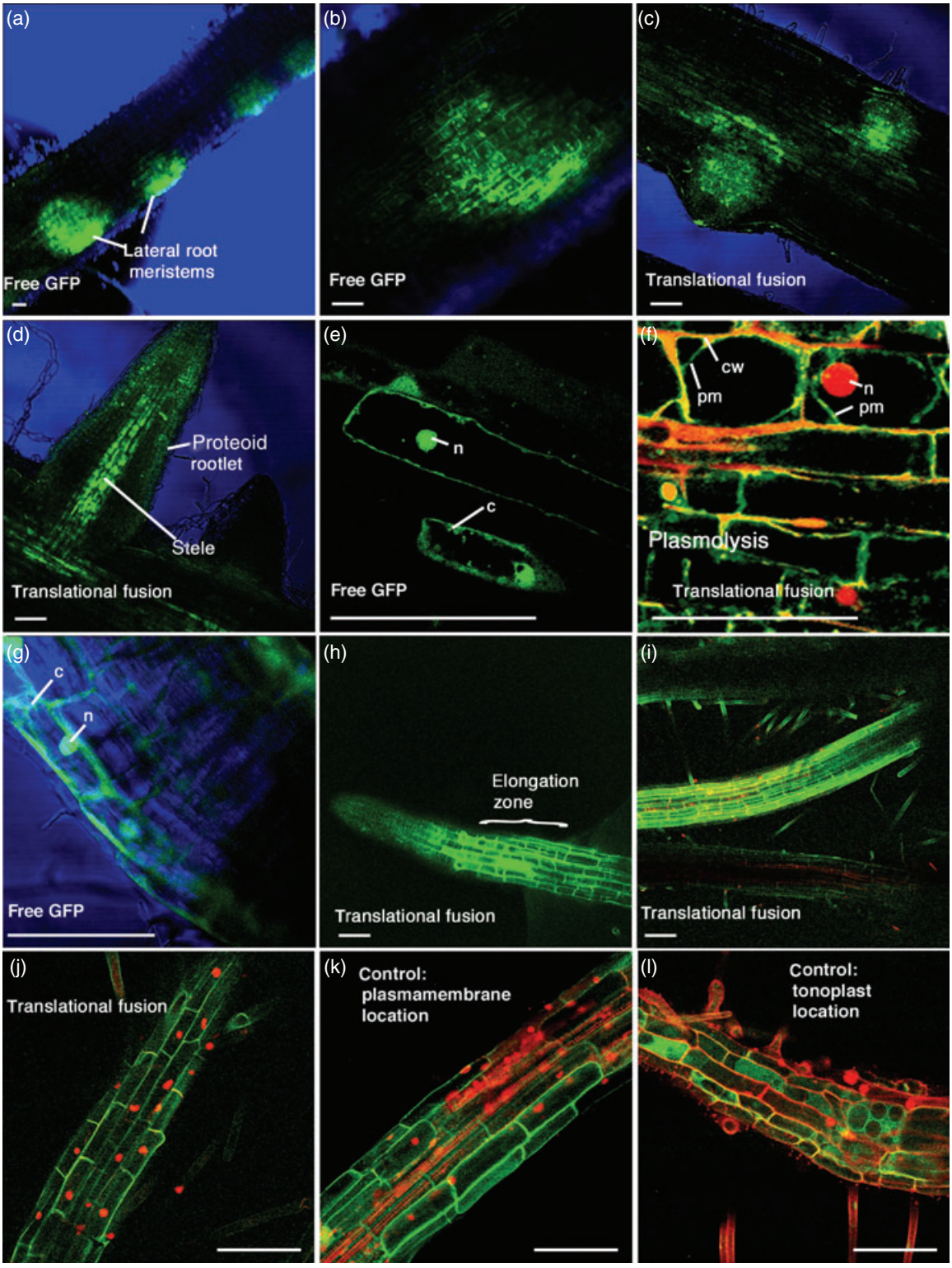
Because LaMATE displayed the highest percentage of identity to FRD3, a T-DNA insertion line of Arabidopsis gene At3g08040 (FRD3) was tested for possible complementation by LaMATE. The T-DNA insertion line SALK_122235 of Arabidopsis gene At3g08040 was identified from the Salk Institute Genomic Analysis Laboratory (SIGnAL) T-DNA insertion collection (http://signal.salk.edu) and obtained from the ABRC. Seeds were grown on germinating soil mix, and heterozygous and homozygous seedlings were identified by PCR. All homozygous seedlings showed significantly delayed growth and development, corresponding to the phenotype previously described for homozygous mutants of this allel (Delhaize, 1996), while heterozygous plants showed no difference compared to the wild type. Two heterozygous plants were transformed with a construct containing the *LaMATE* cDNA under the control of the *LaMATE* promoter. PCR was used to identify homozygous SALK_122235 insertion lines carrying the *LaMATE* cDNA. F1 and F2 seedlings displayed delayed growth and development, compared with heterozygous insertion lines and wild type, indicating that *LaMATE* does not complement the FRD3 mutation in a SALK_122235 T-DNA insertion line.

RNA_i-based silencing of the LaMATE *gene*

DNA sequences that encode a self-complementary region of hairpin (hp) RNA, separated by an intron, have been implicated with effective RNA_i-based gene silencing (Helliwell and Waterhouse, 2003). A 500-bp PCR fragment containing the 5′ coding region of *LaMATE* was inserted via recombination into pHellsgate 8, a vector that has been shown to efficiently generate hpRNA constructs (Helliwell and Waterhouse, 2003; Helliwell *et al.*, 2001). Lupin roots were transformed with the MATE RNA_i construct and with the empty vector as control, respectively. Kanamycin (15 mg l^{-1}) was used to select for the cotransformation of transgenic roots.

RT-PCR was used to compare *LaMATE* expression levels in *A. rhizogenes* transformed roots. Figure 7 shows RT-PCR products derived from proteoid root RNA of four plants independently transformed with *LaMATE* RNA_i ($LaMATE_i$ 1–4) and four plants independently transformed with empty vector pHellsgate8 (control 1–4), respectively. All plants were grown under nutrient deficiency to induce *LaMATE* expression. Plants 1 and 2 were grown under P deficiency and plants 3 and 4 under Fe deficiency. Figure 7(a) (upper panel) shows no detectable *LaMATE* amplification in roots of lupin transformed with the *LaMATE* RNA_i construct, but strong signals in plant roots transformed with the empty vector. A parallel PCR using *ubiquitin* primers indicates similar PCR conditions for all samples (Figure 7a, lower panel).

Inductively coupled plasma (ICP) analysis revealed no significant differences in internal concentrations of P (2 mg ± 0.3 per plant), Fe (0.008 mg ± 0.002 per plant) and Al (0.032 mg ± 0.012 per plant) in *LaMATE*-silenced plants grown under P and Fe deficiency and +Al stress, respectively, compared to empty-vector-transformed control plants (1.8 mg P ± 0.3, 0.010 mg Fe ± 0.002 and 0.043 mg Al ± 0.027 per plant, respectively). Concentrations are



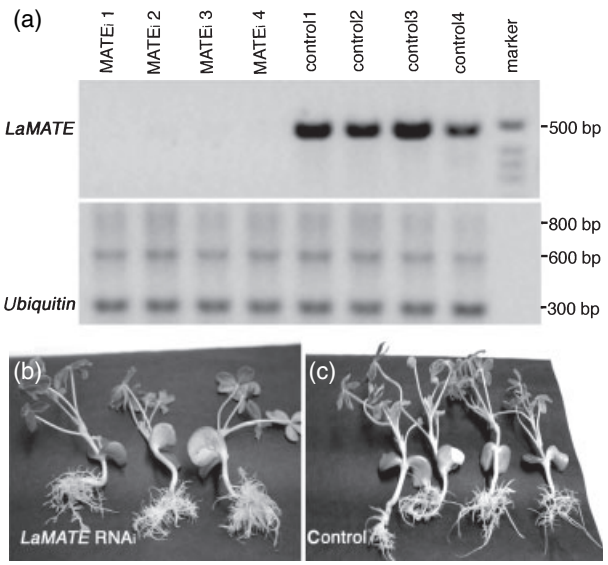


Figure 7. RNAi-based silencing of LaMATE in white lupin.
(a) RT-PCR products derived from proteoid root RNA of four plants independently transformed with *Lupinus albus MULTIDRUG AND TOXIN EFFLUX* (*LaMATE*) RNA_i (LaMATEi 1–4) and four plants independently transformed with the empty vector pHellsgate 8 (control 1–4), respectively. All plants were grown under nutrient-deficient conditions to induce *LaMATE* expression. Plants 1 and 2 were grown under P deficiency, plants 3 and 4 under Fe deficiency. The upper panel shows the result of RT-PCR using a primer pair to amplify a 470-bp fragment of *LaMATE* cDNA. A parallel RT-PCR was performed using a primer pair that amplified *ubiquitin* as control for uniform PCR conditions (lower panel).
(b and c) Phenotypic comparison of the *LaMATE* RNA_i-silenced plants grown under –P indicated less vigor and an approximately 20% reduction of average dry weight, compared with the empty vector-transformed controls. However, root weight was not impaired, resulting in a higher root:shoot ratio in RNA_i-silenced plants (b), compared with the vector-only control plants (c) under –P conditions.

averages of three independent replicates each consisting of two to three whole plants.

Phenotypic comparison displayed that *LaMATE* RNA_i-silenced plants grown under –P indicated had less vigor and an approximately 20% reduction of average dry weight, compared with empty-vector-transformed controls. However, average root weight was not impaired, resulting in a higher root:shoot ratio in LaMATE RNA_i-silenced plants under –P conditions (Figure 7b,c).

Discussion

In this report, we have advanced the fundamental understanding of plant adaptation to –P stress by: (i) developing an *A. rhizogenes*-mediated transformation protocol to generate composite white lupin plants having transgenic roots; (ii) characterizing the full-length *LaMATE* cDNA and isolating the gene including the promoter region, and defining the sequence and exon-intron structure of *LaMATE*; (iii) demonstrating that *LaMATE* transcripts are highly induced in proteoid roots of white lupin under a variety of nutrient stresses; (iv) showing *LaMATE* is expressed in fully elongated proteoid roots and appears to be localized in the plasma membrane; and (v) demonstrating that RNA_i technology effectively silences expression of the *LaMATE* gene in white lupin roots.

Transformation

A major challenge following the identification of P-responsive ESTs in proteoid roots of white lupin (Peñaloza *et al.*, 2002; Uhde-Stone *et al.*, 2003) is to determine the function and regulation of the corresponding genes. White lupin has become an important model organism for the study of plant adaptation to P and Fe- deficiency. However, the use of white lupin as a model organism has been limited by its recalcitrance to transformation. The development of a protocol to generate transgenic white lupin roots provides a system for detailed characterization of P-responsive genes in proteoid roots. The *A. rhizogenes*-mediated transformation of white lupin allows fast and efficient generation of transgenic roots, with the possibility of antibiotic selection for roots expressing a transgene cotransferred in a binary vector.

The morphology of *A. rhizogenes*-induced transgenic roots of white lupin composite plants was similar to normal roots and included the formation of proteoid roots. Transgenic proteoid roots appear to have normal proteoid root morphology, allowing the functional analysis of genes in proteoid roots. However, transgenic roots also developed secondary proteoid roots (proteoid roots forming on lateral proteoid rootlets), which are not

Figure 6. Confocal images of lupin (a–f) and Arabidopsis (g–i) roots, expressing either free enhanced green fluorescence protein (EGFP; a, b, e and g) or a translational *Lupinus albus* MULTIDRUG AND TOXIN EFFLUX (LaMATE)::EGFP fusion (c, d, f, h, i and j).
Both fusions are under the control of the lupin *LaMATE* promoter.
(a and b) The lupin *LaMATE* promoter drives expression of free EGFP in lateral root meristems of P-deficient white lupin.
(c and d) The translational LaMATE::EGFP fusion indicates the tissue-specific location of the LaMATE protein corresponding to the pattern of *LaMATE* gene expression (a and b, and Figure 4), namely in proteoid root meristems and in the stele of proteoid rootlets. (e and f) Subcellular location of the transcriptional (e) and translational (f) EGFP fusion. Free EGFP is found in the nucleus and cytoplasm (e), while the translational LaMATE::EGFP fusion associates with the outline of the cell. Propidium iodide staining of cell walls and nucleus (yellow–red), coupled with plasmolysis experiments (f) indicate that EGFP fluorescence associates with the membrane of plasmolysed cells, rather than with the cell wall.
Free EGFP in Arabidopsis roots is found in the nucleus and cytoplasm (g), while the translational LaMATE::EGFP fusion associates with the outline of the cell (h, i and j). The translational LaMATE::EGFP fusion displays fluorescence in roots at the differentiation and elongation zone, but not at the root tip (h). (i) A region of a young root expressing LaMATE::EGFP in comparison to two root sections not displaying GFP activity. Propidium iodide staining of Arabidopsis expressing LaMATE::EGFP (j) and comparison with a known plasmamembrane-located EGFP fusion (k) and a tonoplast-located EGFP fusion (l; Cutler *et al.*, 2000, ABRCno. CS84758 and CS84727, respectively) confirms the likely localization of the LaMATE protein in the plasmamembrane. Bar size = 100 μm.

typically found in white lupin, but are common in *Banksia* species (Pate and Watt, 2001). These are likely induced by an altered auxin content or sensitivity of *A. rhizogenes* induced transgenic roots (Lemcke and Schmulling, 1998). In addition to the generation of composite plants (transformed root with non-transformed shoot) presented here, we have started the regeneration of whole transgenic lupin plants from transformed root sections, which will be useful for the study of non root-specific genes. Regeneration of whole plants from *A. rhizogenes* transformed roots has been shown for a variety of plants, including tomato (Moghaieb *et al.*, 2004). To this end, small sections of transformed root (approximately 5–10 mm) were placed on Murashige and Skoogs (MS) medium containing sucrose, as well as cytokinin and auxin in a 1:10 ratio. Callus formation was visible after 3 weeks (Figure 1k). Upon transfer to sucrose supplemented MS medium containing cytokinin, calli have begun to differentiate into embryos and roots (Figure 1l).

The multidrug and toxin efflux family

To evaluate the potential for using *A. rhizogenes* mediated root transformation as a tool to study molecular control of proteoid root development and function, we focus on an *LaMATE* gene, which was previously shown to be highly expressed in P-deficient proteoid roots (Uhde-Stone *et al.*, 2003). Currently, about 200 proteins of the MATE family have been sequenced, including representatives from bacteria, archaea, animals, yeast and plants (Hvorup *et al.*, 2003). Only few members of the MATE family have been characterized functionally: these are thought to act as Na^+ (Chen *et al.*, 2002; Morita *et al.*, 2000) or H^+ coupled antiporters (Chen *et al.*, 2002; He *et al.*, 2004; Li *et al.*, 2002; Morita *et al.*, 2000,). Multidrug and toxin efflux proteins have been shown to mediate resistance to a wide range of substances, including antibiotics, aminoglycosides and Cd^{2+} (Chen *et al.*, 2002; Hvorup *et al.*, 2003; Li *et al.*, 2002; Morita *et al.*, 2000).

Many organisms exhibit multiple MATE family paralogues. The largest family of MATE proteins known so far is the MATE protein family in Arabidopsis. This family has at least 56 members that fall into two main groups (Hvorup *et al.*, 2003; Rogers and Guerinot, 2002). The larger group is similar to the yeast and human family members and includes the characterized MATE proteins *Arabidopsis thaliana* detoxification 1 (AtDX1; Li *et al.*, 2002), EDS5 (Nawrath *et al.*, 2002), ALF5 (Diener *et al.*, 2001) and TRANSPARENT TESTA 12 (Debeaujon *et al.*, 2001). FRD3 and FRDL are associated with a smaller group of six MATE proteins in Arabidopsis (Green and Rogers, 2004; Rogers and Guerinot, 2002). The deduced amino acid sequence of *LaMATE* displays highest identity with FRD3 in Arabidopsis (55%). Evidence is accumulating that FRD3 may be involved in shoot iron localization, possibly by loading an iron chelator out of the xylem or apoplastic space into leaf cells (Green and Rogers, 2004). LaMATE shares an internal addition of about 60 amino acids between transmembrane domains 2 and 3 with only a few other MATE proteins, including FRD3.

LaMATE *gene expression*

LaMATE displayed several fold enhanced gene expression in proteoid roots of white lupin under a variety of stresses, namely –P, –Fe, –N, –Mn and +Al stress. Computational analysis of the promoter region identified a number of elements that are frequently found in the promoters of putative *cis*-acting P-responsive genes, including the bindings site recognized by PHR1, a transcriptional factor involved in the regulation of P-responsive genes in Arabidopsis (Rubio *et al.*, 2001), and helix-loop-helix elements (Mukatira *et al.*, 2001). Helix-loop-helix elements were identified in the *LaMATE* promoter region; helix-loop-helix motifs have been implicated for a variety of stress-responses (Smolen *et al.*, 2002). NIT2 elements have originally been identified as activators of N-regulated genes in *Neurospora crassa* (Fu and Marzluf, 1990), indicating a possible role in the N-deficiency response of *LaMATE*. A motif similar to iron-deficiency responsive cis-acting elements, which have been shown to be involved in Fe-responsive expression in transgenic tobacco roots (IDE; Kobayashi *et al.*, 2003), was also present in the *LaMATE* promoter.

The functional diversity of MATE proteins is represented by different gene expression patterns. The FRD3 gene shows twofold induction under iron deficiency in wild-type Arabidopsis. Nawrath *et al.* (2002) characterized a member of the MATE family in Arabidopsis (EDS5) that shows low expression in unstressed plants but is strongly induced by pathogens and UV-C light. AtDTX1, a member of the Arabidopsis MATE family, has been shown to serve as an efflux carrier for plant-derived alkaloids, antibiotics and other toxic compounds including the heavy metal Cd^{2+} (Li *et al.*, 2002). Like *LaMATE*, AtDTX1 was localized in the plasma membrane by using a translational GFP fusion.

The *LaMATE* promoter directed gene expression to proteoid roots of nutrient-stressed white lupin. GUS and EGFP reporter gene fusions displayed a complex expression pattern depending on the developmental stage of proteoid roots. In developed proteoid rootlets of nutrient stressed plants, *LaMATE* expression is found primarily in the stele and, with further development, in all root tissues, except for a zone adjacent to the root tip. The localization of *LaMATE* expression in lupin resembles that of FRD3 in Arabidopsis, which was found to be expressed in the pericycle and cells internal to the pericycle and surrounding the vascular tissue (Green and Rogers, 2004). High expression at the pericycle/endodermis could indicate involvement of xylem loading, or phloem unloading, of a small organic molecule.

RNA$_i$-based gene silencing

We have recently been successful in silencing a lupin acid phosphatase (data not shown; J. Li, C. Uhde-Stone, D. Allan and C. Vance, University of Minnesota, St Paul). Functional characterization of the polyploid white lupin genes would be greatly facilitated by stable silencing of specific genes. RNA$_i$ has been demonstrated to suppress the expression of a wide range of genes in a variety of plants (reviewed by Waterhouse and Helliwell, 2003). Here, we have shown that RNA$_i$ significantly silenced expression of the *LaMATE* gene in transgenic roots of white lupin. Phenotypic characterization of the LaMATE RNA$_i$-silenced plants grown under −P indicated less vigor and an approximately 20% reduction of average dry weight, compared with vector-only transformed control plants. However, root dry weight was not reduced, resulting in a higher root:shoot ratio in the LaMATE RNA$_i$-silenced plants. An increased root:shoot ratio is a known response of plants to P-deficiency (Vance *et al.*, 2003). This finding indicates that sensing of −P is not impaired in LaMATE-silenced lupin plants. The observed phenotype in lupin resembles that of the FRD3 phenotype of smaller plants in Arabidopsis (Delhaize, 1996). However, in contrast to the metal accumulating FRD3 mutant, ICP analysis revealed no significant differences in internal concentrations of LaMATE-silenced plants grown under −P, −Fe and +Al, respectively, compared to empty-vector transformed control plant. This finding, together with the observation that LaMATE does not complement the FRD3 phenotype, indicates a function of LaMATE different from that of FRD3. Experiments are ongoing to further elucidate the function of LaMATE in response to nutrient stresses.

Experimental procedures

Plant material

White lupin (*Lupinus albus* L. var Ultra) was grown in the growth chamber in sand culture under growth conditions as previously described (Gilbert *et al.*, 2000; Johnson *et al.*, 1996a). For transformation experiments, Arabidopis seedlings ecotype Columbia were grown in germinating soil mix. For expression analysis under nutrient deficiency, transgenic Arabidopsis seeds (F1 generation) were grown for 7 days on Km50; Km-resistant seedlings were transferred and grown for 10 and 20 days, respectively, on slanted 0.6% agarose plates containing 1× Hoagland solution adjusted to +P, −P, low P (25 μM), −Fe and −N.

Agrobacterium rhizogenes-*mediated transformation of white lupin*

Surface sterilized seeds (3 min sterilization in 1% bleach, followed by several rinses of sterile water) were germinated in the dark. When emerging radicles reached a length of approximately 10 mm, tip sections of approximately 3 mm were removed with a sterile scalpel. The radicle was inoculated with the appropriate *A. rhizogenes* strain (derivatives of *A. rhizogenes* strain A4T; Quandt *et al.*, 1993) and grown on trypton-yeast (TY) plates containing 100 mM acetosyringone and 1% glucose. Six to eight seedlings were placed on 200 ml slanted agarose (0.6% in 1× Hoagland solution, 15 mg l^{-1} Kanamycin) in clear 222 × 222 mm Q-trays with covers (Genetix USA Inc., Boston, MA, USA). Plates were then placed vertically in a growth chamber at 18 to 20°C for 14 h photoperiods with a light intensity of about 200 PAR (measured in E s^{-1} m^{-2}). Plants that showed hairy root development after about 3 weeks (about 50%) were transferred to fresh plates and incubated for another 1–4 weeks.

Generation of transgenic Arabidopsis

To analyze the expression profile of *LaMATE* in Arabidopsis, we generated heterozygous F1 transgenic Arabidopsis carrying transcriptional *LaMATE*::GUS and *LaMATE*::EGFP fusions, and translational MATE::GUS fusion under the control of the *LaMATE* promoter, respectively. Three independently generated lines were analyzed for each construct. Transgenic Arabidopsis was generated via *A. tumefaciens*-mediated transformation, using the floral dip/vacuum infiltration method (Bent *et al.*, 1994).

RNA gel blots and DNA gel blots

RNA for gel blot analysis and genomic DNA were isolated as described previously (Liu *et al.*, 2001). RNA gel blots were performed in replicate.

RT-PCR

RNA for RT-PCR was isolated from independently grown plants using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Quantitation of the transcripts was performed using two-step RT-PCR following the manufacturer's directions (Ambion, Austin, TX, USA, and Invitrogen) using poly thymine deoxynucleotide (dT) primer. The two primer pairs used in the parallel PCR reactions were: LaMATE1 5′-GGATTCCAAGTTTGCCTTCAAGT-3′ and LaMATE 5′-GTT3CCTGTTCCCATTCTCCAAAC-3′. This primer pair amplifies a 470 bp from the 3′ coding region of LaMATE. This region has not been used for the MATE RNA$_i$ constuct. The primer pair used as control was *ubiquitin1* 5′-TCTTTGTGAAGACCCTCACC-3′ and *ubiquitin2* 5′-CTGCTGGTCCGGAGGAATG-3′. PCR was performed for 26 cycles (94°C for 1 min, 56°C for 30 sec, 72°C for 30 sec).

Screening of a lupin genomic library

A partially genomic library of white lupin in a γ DASH II vector has been constructed and described previously (Liu *et al.*, 2001). After amplification, plaque filters were hybridized with a random primer-labeled *LaMATE* cDNA fragment. Single plaques were purified after two rounds of hybridization and the insert was then subcloned in pBluescript vector (Stratagene, La Jolla, CA, USA) for restriction mapping and sequencing.

Sequencing and computational analyses

Sequencing was carried out at the Advanced Genetic Analysis Center (St Paul, MN, USA). Sequences were processed with the UWGCG program (Devereux and Smithies, 1984).

Construction of transcriptional and translational reporter gene fusions

A 2-kb portion of the genomic 5′ upstream region of *LaMATE* was amplified using the following primer pair introducing an upstream *Hin*dIII and a downstream *Sal*I restriction site, respectively. The left primer was (*Hin*dIII) 5′-TATAAGCTTCATACTGGTCAAAACTGGTCG-3′; the right primer was (*Sal*I) 5′-TATGTCGACCCATTCTCTGCCATGTGAAAC-3′. The amplified product was ligated into pGEMTeasy (Promega, Madison, WI, USA) and subcloned as a 2-kb *Hin*dIII/*Sal*I fragment into pBI101.2 (BD Biosciences Clontech, Palo Alto, CA, USA) and pBI101.2-EGFP, respectively. The vector pBI101.2 carries a promoter-less GUS cassette in the *Agrobacterium* binary plasmid vector pBIN 19 (Bevan, 1984; Jefferson, 1989). Plasmid pBI101.2-EGFP was constructed by amplifiying the EGFP gene of plasmid pEGFP (BD Biosciences Clontech) using the following primer pair introducing an upstream *Xma*I and a downstream *Sst*I restriction site, respectively. The left primer was (*Xma*I) 5′-TATCCCGGGTACCGGTCGCCAC-3′; the right primer was (*Sst*I) 5′-TATGAGCTCCAGTTGGAATTCTAGAGTCGCG-3′. The amplified product was ligated into pGEMTeasy, the sequence confirmed by full-length sequencing, and subcloned as a 700-kb *Xma*I/*Sst*I fragment into pB101.2., replacing a 1.7-kb *Xma*I/*Sst*I fragment containing the GUS reporter gene, but not affecting the downstream polyadenylation signal.

For construction of a translational MATE–EGFP fusion, the 1.6-kb coding region of the *LaMATE* cDNA was amplified except for the stop codon, introducing an upstream *Sal*I and a downstream *Xma*I restriction site. The left primer was (*Sal*I) 5′-ATAGTCGACCATGGCAGAGAATGGCACTG-3′; the right primer was (*Xma*I) 5′-ATACCCGGGGCACAGACATTGGGTGGCTTC-3′. The amplified product was ligated into pGEMTeasy. The PCR product was confirmed by full-length sequencing and the 1.6-kb *Sal*I/*Xma*I fragment was inserted in-frame with EGFP in the transcriptional *LaMATE*-promoter::EGFP fusion in pBI101.2 between the *LaMATE*-promoter and the EGFP fusion. In-frame transitions were confirmed by sequencing.

Histochemical and fluorometric GUS assays

For histochemical GUS activity detection, samples were incubated overnight at 37°C in GUS assay buffer, using 5-bromo-4-chloro-3-indoyl glucuronide as a substrate (Jefferson, 1989). GUS staining was observed using a stereoscopic zoom microscope (SMZ800; Nikon, Melville, NY, USA) and images were taken with a DXM1200 digital camera (Nikon).

For quantitative fluorometric MUG assays, proteoid roots of different developmental stages were harvested from independently transformed plants grown under +P, –P, –N and –Fe, respectively, and quantification was performed as described previously (Trepp *et al.*, 1999).

Fluorescence microscopy

Confocal microscopy was performed using an MRC 1024 laser confocal microscope (Bio-Rad, Hercules, CA, USA) with a Krypton/Argon laser source at the C.B.I. Imaging Center (St Paul, MN, USA), and an LSM-510 META confocal microscope (Zeiss, Jena, Germany) at the College of Natural Resource's Biological Imaging Facility at the University of California (Berkeley, CA, USA). Optical planes were scanned with the 488-nm laser ray, using a 505–550 nm barrier filter to detect GFP fluorescence. For imaging of GFP and propidium iodide staining, multitracking was performed using a second laser with a 543-nm laser ray and a 570-nm barrier filter. Plasmolysis experiments were performed by placing plant material in 0.6 M glucose for 30 min to 3 h. Roots were stained with propidium iodide in a concentration of 10 μg ml^{-1} before observation.

Complementation of an Arabidopsis T-DNA insertion line

Seeds of T-DNA insertion line SALK_122235 were grown on germinating soil mix, and heterozygous and homozygous seedlings were determined by PCR using the following three primers: left border primer LBa1 5′-GGTTCACGTAGTGGGCCATCG-3′, LP SALK_122235 5′-TACCATGGAGTATGCAGTGTAAGCAA-3′ and RP SALK_122235 5′-TGGAAGGTTCCTTAAAAATGGTACGT-3′.

The *LaMATE*-promoter::GUS fusion in pB101.2 was used as basis for the construction of *LaMATE* cDNA under control of the MATE promoter. The MATE cDNA was amplified using the following primer pair: *LaMATE* cDNA *Sal*I 5′-ATAGTCGACCATGGCAGAGAATGGCACTG-3′ and *LaMATE* cDNA SstI 5′-ATAGAGCTCATCACACAGACATTGGGTGG-3′. The amplified product was ligated into pGEMTeasy and, after the PCR product was confirmed by full-length sequencing, the *Sal*I-*Sst*I fragment containing the MATE cDNA was used to replace the GUS-reporter gene upstream of the Nos-terminator.

Gene silencing in white lupin

To amplify a 500-bp PCR product of the 5′ region of *LaMATE*, flanked by attachment sites (attB1 and attB2), the following primer pair was used: MATE attB1 5′-ggggacaagtttgtacaaaaaagcaggctTGCTGCTGATCCTCTTGCTTC-3′ and MATE attB2 5′-ggggaccactttgtacaagaaagctgggtGGCCAAGGAGTAAGCCAAAAA-3′. The attachment sites (shown in non-capital letters) allowed the PCR product to recombine into the attachment site of pDONR201, mediated by the GATEWAY™ BP CLONASE™ (Invitrogen). Subsequently, the fragment of interest was recombined into pHellsgate8 (Helliwell and Waterhouse, 2003), mediated by the LR Clonase™ Enzyme Mix (Invitrogen). PCR and restriction analysis confirmed correct orientation of the gene fragments and intron. This construct, as well as the empty pHellsgate 8 vector were used for *A. rhizogenes* transformation of white lupin.

Phenotypic analysis of LaMATE *RNA$_i$-silenced mutants*

LaMATE RNA_i-silenced white lupin and empty vector-transformed controls were grown under normal nutrient conditions and under –P, –Fe and +Al stress, respectively. Seven weeks after *A. rhizogenes* inoculation, plants were harvested, fresh and dry weight was determined, and dried plants (after 2 days at 80°C) were pooled in groups of two to three per analysis. Replicates of pooled plants were analyzed by ICP analysis at the University of Minnesota Soil testing laboratory, St Paul campus.

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

Accession numbers

The GenBank accession number for *LaMATE* cDNA is AY631874 and for the *LaMATE* gene is AY631873.

Acknowledgements

The authors wish to thank Lynn Hartweck, John Ward and Anke Reinders for advice and help with growing and transforming Arabidopsis. We also gratefully acknowledge Sergey Ivashuta for help with the RNA_i constructs. We further wish to thank Steve Ruzin at the College of Natural Resource's Biological Imaging Facility at the University of California, Berkeley. We also wish to thank students at California State University East Bay who assisted on this project, especially Saptaparnee Mukherjee and Mousumi Rath. This research was supported in part by US Department of Agriculture National-Research Initiative Competitive Grant no. USDA-CSREES/2002-35100-12206.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Schematic representation of the intron/exon structure of the *Lupinus albus MULTIDRUG AND TOXIN EFFLUX*, (*LaMATE*) gene and location of putative *cis*-binding elements in the *LaMATE* promoter region.

This material is available as part of the online article from http://www.blackwell-synergy.com

References

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J. and Staskawicz, B.J. (1994) RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* **265**, 1856–1860.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* **12**, 8711–8721.

Blackwell, T.K. and Weintraub, H. (1990) Differences and similarities in DNA-inding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **250**, 1104–1110.

Boisson-Dernier, A., Chabaud, M., Garcia, F., Becard, G., Rosenberg, C. and Barker, D.G. (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant Microbe Interact.* **14**, 695–700.

Brown, M.H., Paulsen, I.T. and Skurray, R.A. (1999) The multidrug efflux protein NorM is a prototype of a new family of transporters. *Mol. Microbiol.* **31**, 394–395.

Cangelosi, G.A., Ankenbauer, R.G. and Nester, E.W. (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc. Natl Acad. Sci. USA* **87**, 6708–6712.

Chen, J., Morita, Y., Huda, M.N., Kuroda, T., Mizushima, T. and Tsuchiya, T. (2002) VmrA, a member of a novel class of Na(+)-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. *J. Bacteriol.* **184**, 572–576.

Cutler, S.R., Ehrhardt, D.W., Griffitts, J.S. and Somerville, C.R. (2000) Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *Proc. Natl Acad. Sci. USA* **97**, 3718–3723.

Debeaujon, I., Peeters, A.J., Leon-Kloosterziel, K.M. and Koornneef, M. (2001) The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. *Plant Cell*, **13**, 853–871.

Delhaize, E. (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. *Plant. Physiol.* **111**, 849–855.

Devereux, J.H.P. and Smithies, O. (1984) Sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**, 387–395.

Diener, A.C., Gaxiola, R.A. and Fink, G.R. (2001) Arabidopsis ALF5, a multidrug efflux transporter gene family member, confers resistance to toxins. *Plant Cell*, **13**, 1625–1638.

Dinkelaker, B., Romheld, V. and Marschner, H. (1989) Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Environ.* **12**, 285–292.

Fu, Y.H. and Marzluf, G.A. (1990) NIT-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence specific DNA-binding protein. *Proc. Natl Acad. Sci. USA* **87**, 5331–5335.

Gardner, W.K., Parbery, D.G. and Barber, D.A. (1982) The acquisition of phosphorus by *Lupinus albus* L. I. Some characteristics of the soil/root interface. *Plant Soil*, **68**, 19–32.

Gilbert, G.A., Knight, J.D., Vance, C.P. and Allan, D.L. (1999) Acid phosphatase activity in phosphorus-deficient white lupin roots. *Plant Cell Environ.* **22**, 801–810.

Gilbert, G.A., Knight, J.D., Vance, C.P. and Allan, D.L. (2000) Proteoid root development of phosphorus deficient lupin is mimicked by auxin and phosphonate. *Ann. Bot.* **85**, 921–928.

Green, L. and Rogers, E. (2004) FRD3 controls iron localization in Arabidopsis. *Plant Physiol.* **136**, 2523–2531.

He, G.X., Kuroda, T., Mima, T., Morita, Y., Mizushima, T. and Tsuchiya, T. (2004) An H(+)-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. *J. Bacteriol.* **186**, 262–265.

Helliwell, C. and Waterhouse, P. (2003) Constructs and methods for high-throughput gene silencing in plants. *Methods*, **30**, 289–295.

Helliwell, C.A., Chin-Atkins, A.N., Wilson, I.W., Chapple, R., Dennis, E.S. and Chaudhury, A. (2001) The Arabidopsis AMP1 gene encodes a putative glutamate carboxypeptidase. *Plant Cell*, **13**, 2115–2125.

Hvorup, R.N., Winnen, B., Chang, A.B., Jiang, Y., Zhou, X.F. and Saier, M.H., (2003) The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. *Eur. J. Biochem.* **270**, 799–813.

Jefferson, R.A. (1989) The GUS reporter gene system. *Nature* **342**, 837–838.

Johnson, J.F., Allan, D.L., Vance, C.P. and Weiblen, G. (1996a) Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*, contribution to organic acid exudation by proteoid roots. *Plant Physiol* **112**, 19–30.

Johnson, J.F., Vance, C.P. and Allan, D.L. (1996b) Phosphorus deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant. Physiol.* **112**, 31–41.

Kobayashi, T., Nakayama, Y., Itai, R.N., Nakanishi, H., Yoshihara, T., Mori, S. and Nishizawa, N.K. (2003) Identification of novel cis-acting elements, IDE1 and IDE2, of the barley IDS2 gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. *Plant J.* **36**, 780–793.

Lemcke, K. and Schmulling, T. (1998) Gain of function assays identify non-rol genes from *Agrobacterium rhizogenes* TL-DNA that alter plant morphogenesis or hormone sensitivity. *Plant J.* **15**, 423–433.

Li, L., He, Z., Pandey, G.K., Tsuchiya, T. and Luan, S. (2002) Functional cloning and characterization of a plant efflux carrier for multidrug and heavy metal detoxification. *J. Biol. Chem.* **277**, 5360–5368.

Liu, J., Uhde-Stone, C., Li, A., Vance, C.P. and Allan, D.L. (2001) A phosphate transporter with enhanced expression in proteoid roots of white lupin (*Lupinus albus* L.). *Plant Soil*, **237**, 257–266.

Massonneau, A., Langlade, N., Leon, S., Smutny, J., Vogt, E., Neumann, G. and Martinoia, E. (2001) Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status. *Planta*, **213**, 534–542.

Mitaku, S. and Hirokawa, T. (1999) Physicochemical factors for discriminating between soluble and membrane proteins: hydrophobicity of helical segments and protein length. *Protein Eng.* **12**, 953–957.

Moghaieb, R.E., Saneoka, H. and Fujita, K. (2004) Shoot regeneration from GUS-transformed tomato (*Lycopersicon esculentum*) hairy root. *Cell Mol. Biol. Lett.* **9**, 439–449.

Morita, Y., Kataoka, A., Shiota, S., Mizushima, T. and Tsuchiya, T. (2000) NorM of *Vibrio parahaemolyticus* is an Na(+)-driven multidrug efflux pump. *J. Bacteriol.* **182**, 6694–6697.

Mukatira, U.T., Liu, C., Varadarajan, D.K. and Raghothama, K.G. (2001) Negative regulation of phosphate starvation-induced genes. *Plant Physiol.* **127**, 1854–1862.

Nawrath, C., Heck, S., Parinthawong, N. and Metraux, J.P. (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. *Plant Cell*, **14**, 275–286.

Neumann, G. and Martinoia, E. (2002) Cluster roots – an underground adaptation for survival in extreme environments. *Trends Plant Sci* **7**, 162–167.

Neumann, G., Massonneau, A., Martinoia, E. and Romheld, V. (1999) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta*, **208**, 373–382.

Neumann, G., Massonneau, A., Langlade, N., Dinkelaker, B., Hengeler, C., Romheld, V. and Martioia, E. (2000) Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). *Ann. Bot.* **85**, 909–919.

Pate, J. and Watt, M. (2001) Roots of *Banksia* spp. (Proteaceae) with special reference to functioning of their specialized root clusters. In *Plant roots: the hidden half, 3rd edn.* (Waisel Y., Eshel A. and Kafkafi U., eds). New York: Marcel Dekker Inc., pp. 989–1006.

Penaloza, E., Gutierrez, A., Martinez, J., Munoz, G., Bravo, L.A. and Corcuera, L.J. (2002) Differential gene expression in proteoid root clusters of white lupin (*Lupinus albus*). *Physiol. Plant.* **116**, 28–36.

Quandt, H.-J., Puehler, A. and Broer, I. (1993) Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. *Mol. Plant Microbe Interact.* **6**, 699–706.

Raghothama, K.G. (1999) Phosphate acquisition. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 665–693.

Rogers, E.E. and Guerinot, M.L. (2002) FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. *Plant Cell*, **14**, 1787–1799.

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A. and Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes Dev.* **15**, 2122–2133.

Runge–Metzger, A. (1995) Closing the cycle: obstacles to efficient P management for improved global security. In *Phosphorus in the Global Environment* (Ticssen, H., ed.). Chichester: John Wiley and Sons Ltd, pp. 27–42.

Smolen, A.G., Pawlowski, L., Wilensky, S.E. and Bender, J. (2002) Dominant alleles of the basic helix-loop-helix transcription factor ATR2 activate stress-responsive genes in Arabidopsis. *Genetics* **161**, 1235–1246.

Tang, Z., Sadka, A., Morishige, D.T. and Mullet, J.E. (2001) Homeodomain leucine zipper proteins bind to the phosphate response domain of the soybean VspB tripartite promoter. *Plant Physiol.* **125**, 797–809.

Trepp, G.B., van de Mortel, M., Yoshioka, H., Miller, S.S., Samac, D.A., Gantt, J.S. and Vance, C.P. (1999) NADH-glutamate synthase in alfalfa root nodules. Genetic regulation and cellular expression. *Plant Physiol.* **119**, 817–828.

Tusnady, G.E. and Simon, I. (2001) Topology of membrane proteins. *J. Chem. Inf. Comput. Sci.* **41**, 364–368.

Uhde-Stone, C., Zinn, K.E., Ramirez-Yanez, M., Li, A., Vance, C.P. and Allan, D.L. (2003) Nylon filter arrays reveal differential gene expression in proteoid roots of white lupin in response to phosphorus deficiency. *Plant Physiol.* **131**, 1064–1079.

Vance, C.P., Uhde-Stone, C. and Allan, D. (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a on-renewable resource. *Tansley Rev.* **157**, 423–447.

Waterhouse, P.M. and Helliwell, C.A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nat. Rev. Genet.* **4**, 29–38.

Yanagisawa, S. (2004) Dof domain proteins: plant-specific transcription factors associated with diverse phenomena unique to plants. *Plant Cell Physiol.* **45**, 386–391.